Design, Synthesis and Bioevaluation of Novel Bile Acid Conjugates

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SYNOPSIS

The thesis entitled *"Design, Synthesis and Bioevaluation of Novel Bile Acid Conjugates"* deals mainly with the synthesis, characterization and bioevaluation of deoxycholic acid-chiral amino alcohols/chalcones/thiadiazole conjugates. The work presented in the thesis has been described under four chapters, and a brief description is given below.

Chapter 1: Introduction

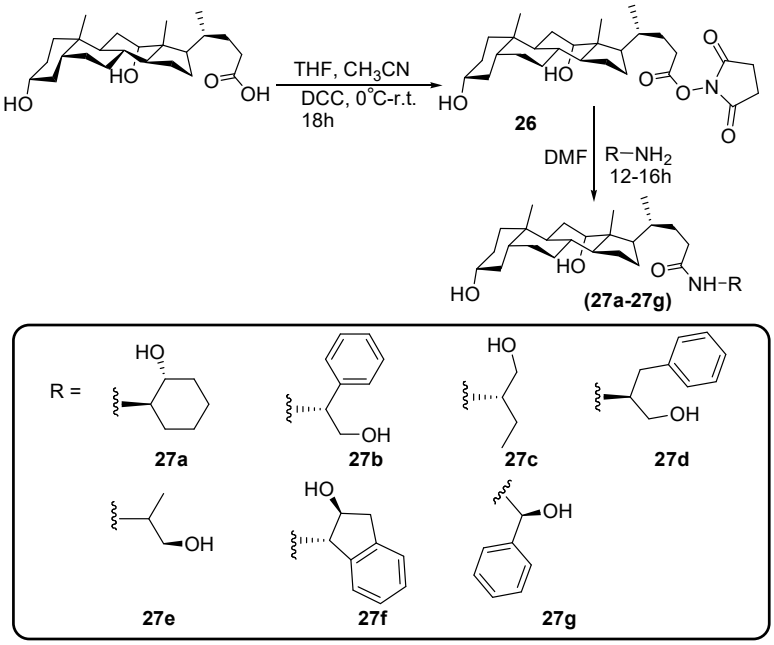
First chapter gives a brief introduction to the bile acid and bile acid-based conjugates and their pharmaceutical/physicochemical applications. Nature continues to be the main source of motivation for synthetic chemists in their expedition to prepare novel conjugates, which can have different physical, biological and medicinal properties. Steroids are a group of lipids whose chemical structure is characteristic of the biological world. Steroid structures have gradually become to a greater extent important in various fields, such as pharmacology, bio mimetic, supramolecular chemistry, and nanotechnology. Recently, literature report illustrated pharmacological applications of bile acid derivatives viz. antibacterial activity[1-5], antifungal [6-7], antitumor [8] or as drug carriers [9].

The synthesis of hybrid molecules by the combination of two biologically active moieties has been a successful strategy for the development of novel therapeutic agents with excellent potency and lower side effects. With this in mind and using common bile acid-based conjugates, we intend to synthesize bile acid-chiral amino alcohol/chalcone/thiadiazole amide conjugates. Chapter 2-4 describes the design, synthesis, characterization and evaluation of bile acid conjugates with the intention to develop innovative lead molecules.

Chapter 2: Design, Synthesis, Characterization and Anti-bacterial Activity Evaluation of Deoxycholic acid –Chiral Amino Alcohol Conjugates

A common feature of bile acid-derived antimicrobials is their potential to exhibit a facially amphiphilic nature due to both a hydrophobic face and a hydrophilic face. Bile acids have been believed extremely helpful in the preparation of novel pharmaceutical drugs because of their intrinsic chemical and biological properties[10-11]. Structural indiscretion, the reactivity of bile acids and their various native biological activities make these scaffolds a fascinating and promising starting material for the synthesis of new compounds with precious biological activities[12].

Numerous synthetic bile acid derivatives have been recognized for its various biological activities. Among these, bile acid amides have appeared as an eye-catching antibacterial agent. In view of this and taking a lead from the existing bile acid-amide conjugates, we planned to synthesize bile acid-chiral amino alcohol conjugates. Novel deoxycholic acid-chiral amino alcohol conjugates were synthesized, from conjugation of deoxycholic acid-NHS ester with amino alcohols at C-24 position of deoxycholic acid to yield deoxycholic acid-amino alcohol conjugates. We prepared deoxycholic acid-amino alcohol conjugates via the synthetic route depicted in **Scheme 2.1**. All the synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR and mass-spectroscopy.

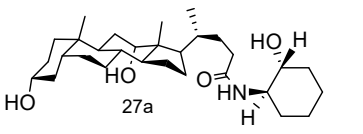


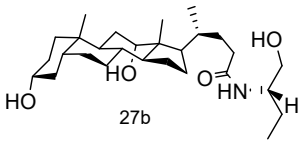
Scheme 2.1: Synthesis of Deoxycholic acid (DAC)-amino alcohol conjugates

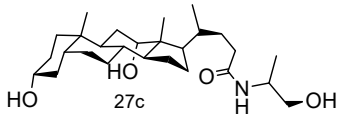
The entire synthesized deoxycholic acid-amino alcohol conjugates were evaluated for their antibacterial activity against *S. aureus* and *E. coli* bacteria. From the antibacterial data (**Table 2.1**), it was observed that compound **27a** (N-(3α-Hydroxy-5β-cholan-24-oyl)-(*R*)-2-aminocyclohexanol) showed comparatively better results against *S. aureus* with a MIC value of 15 µg/mL and *E. coli* with a MIC value of 45 µg/mL. Amongst all evaluated compounds in this study, the derivative **27c** (N-(3α-Hydroxy-5β-cholan-24-oyl)-(*S*)-(+)-1-Amino-2-propanol) was inactive against both bacterial strains evaluated with MIC ≥120 µg/mL. Compound **27g** (N-(3α-Hydroxy-5β-cholan-24-oyl)-(*R*)-(−)-2-Amino-1-phenylethanol),

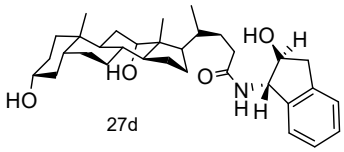
having a (*R*)-(−)-2-Amino-1-phenylethanol at C-24, showed more activity than compounds **27b-27f** against *S. aureus*. The compounds having (*R*)-2-aminocyclohexanol and (1*R*, 2*R*)-(−)-trans-1-Amino-2-indanol moiety at C-24 (**27a** and **27d**) were the most active compounds against the *E. coli*. The compounds **27b** and **27g** were significantly less active against the *E. coli* strain still keeping the potential to inhibit the growth of the *S. aureus* strain. This variation was, may be, due to the impotency of compounds **27b** and **27g** to pass through the surface membrane of the Gram-negative bacteria. A little change in linker size and position of alcohol in chiral amino alcohol-deoxycholic acid conjugates influences the activities of chiral amino alcohol-DCA conjugates against both *S. aureus* and *E. coli* (**Table 2.1**). Compounds **27a** and **27d** were demonstrated potent antimicrobial activity against both *E. coli* and *S. aureus*.

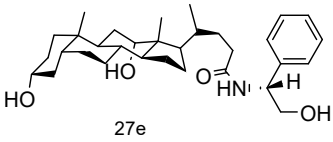
Table 2.1: Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of deoxycholic acid-amino alcohol conjugates against Gram positive (*Staphylococcus aureus* ATCC 25923) and Gram negative bacterial (*Escherichia coli* ATCC 25922)

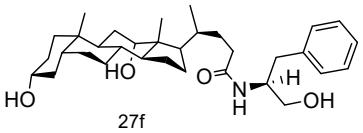


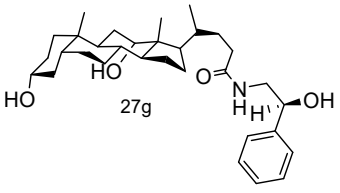












Compound No	MIC (µg/mL)		MBC (µg/mL)	
	S. aureus	E. Coli	S. aureus	E. Coli
27a	15	45	60	90
27b	60	120	180	200
27c	>120	120	200	200
27d	30	60	90	120
27e	120	>120	200	200
27f	>180	60	200	>200
27g	45	120	200	200
Ampicillin	15	4	-	-

This is evident from results that the chirality of amino alcohol played a crucial role in deoxycholic acid-amino alcohol conjugates in inhibiting the growth of bacterial cells. All the

synthesized deoxycholic acid-amino alcohol conjugates were also executed for minimum bactericidal concentration (MBC) values against *S. aureus* and *E. coli* bacteria (**Table 2.1**). Compound **27a** exhibited MBCs of 60 and 90 µg/mL against *S. aureus* and *E. coli* and **27d** demonstrate MBC at 90 and 120 µg/mL for the strain *S. aureus* and *E. coli*. However, compounds **27b-27c** and **27e-27g** appear to exhibit complete bacterial inhibition at higher concentrations. Compounds **27a** and **27d** were the better MBCs among all tested compounds.

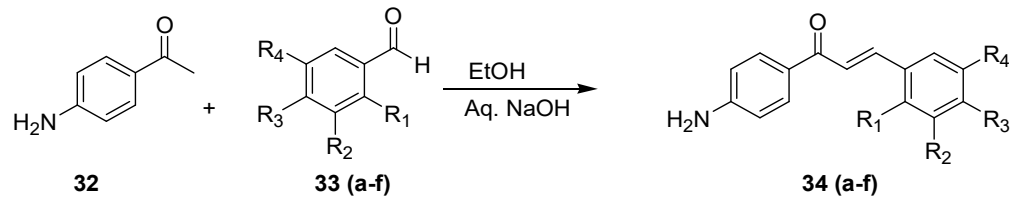
Compound **27a** showed the maximum activity against *S. aureus* (zone of inhibition is about 16 mm) and *E. coli* (zone of inhibition is about 14 mm) with MIC value of 45 µg/mL against *E. coli* and 15 µg/mL against *S. aureus*. The structure-activity relationship result established that antibacterial activities of the deoxycholic acid-amino alcohol conjugates were importantly dependent upon the substituents of amino alcohols and these associations point towards the further design and developments of new deoxycholic acid-amino alcohol conjugates with superior anti-bacterial activities.

Chapter 3: Design and Synthesis of Deoxycholic Acid-Chalcone Conjugates as Potential Anticancer Agents

One of the main research goals today is to prepare new steroid molecules with simpler chemical reactions and easier preparation. This would also provide a platform to approach the synthesis of new drugs for tackling important biological problems. The cholesterol-chalcone conjugates showed significant antibacterial activity but poor cytotoxicity and antifungal activity[13]. Synthetic bile acid-chalcone and bile acid thiazide conjugates were evaluated for antibacterial activity[14] There are reports that sterol-chalcone conjugates are used as antibacterial agents[15]. Taking this into consideration of the previous report, hereby we outline the design and synthesis of novel bile acid-chalcone conjugate appended via an amide linkage. N-(4-Acetylphenyl)-3α, 7α, 12α-trihydroxy-5β-cholan-24-amide is prepared by coupling deoxycholic acid and 4-aminoacetophenone under standard peptide chemistry methods. The objective of this study was the investigation of the effect of deoxycholic acid on the efficacy of chalcone.

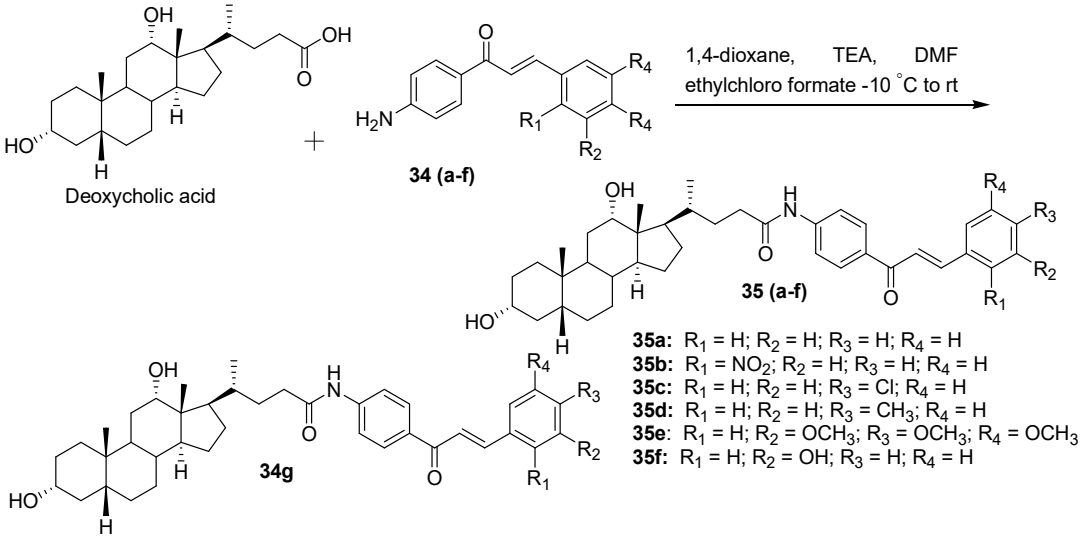
The synthesis of deoxycholic acid-chalcone conjugates has been demonstrated in **Scheme 3.1** and **3.2**. The 4-amino chalcone derived building blocks **34a–34g** were synthesized in 80–92% overall yields following the available procedure via the reaction of 4-amino acetophenone with respective aromatic aldehydes. Briefly, deoxycholic acid-chalcone conjugates synthesized as follows. Ethyl chloroformate was added dropwise to a cooled solution of the deoxycholic acid and triethylamine in 1,4-dioxane. After 20 min, 4-amino

chalcone derivatives and triethylamine were added to the reaction mixture as a solution in DMF. After the completion, the crude product was purified using silica gel chromatography with ethyl acetate and hexane as eluent to furnish the deoxycholic acid chalcone conjugates **35a–35g** in 60–75% yields. The structures of the synthesized bile acid-chalcone conjugates were characterized employing ^{1}H & ^{13}C NMR and mass spectroscopic studies.



34a: R_1 = H; R_2 = H; R_3 = H; R_4 = H
34b: R_1 = NO_2; R_2 = H; R_3 = H; R_4 = H
34c: R_1 = H; R_2 = H; R_3 = Cl; R_4 = H
34d: R_1 = H; R_2 = H; R_3 = CH_3; R_4 = H
34e: R_1 = H; R_2 = OCH_3H; R_3 = OCH_3; R_4 = OCH_3
34f: R_1 = OH; R_2 = H; R_3 = H; R_4 = H

Scheme 3.1: Synthesis of amino-derived chalcones



35a: R_1 = H; R_2 = H; R_3 = H; R_4 = H
35b: R_1 = NO_2; R_2 = H; R_3 = H; R_4 = H
35c: R_1 = H; R_2 = H; R_3 = Cl; R_4 = H
35d: R_1 = H; R_2 = H; R_3 = CH_3; R_4 = H
35e: R_1 = H; R_2 = OCH_3; R_3 = OCH_3; R_4 = OCH_3
35f: R_1 = H; R_2 = OH; R_3 = H; R_4 = H

Scheme 3.2: Synthesis of amino-derived chalcones

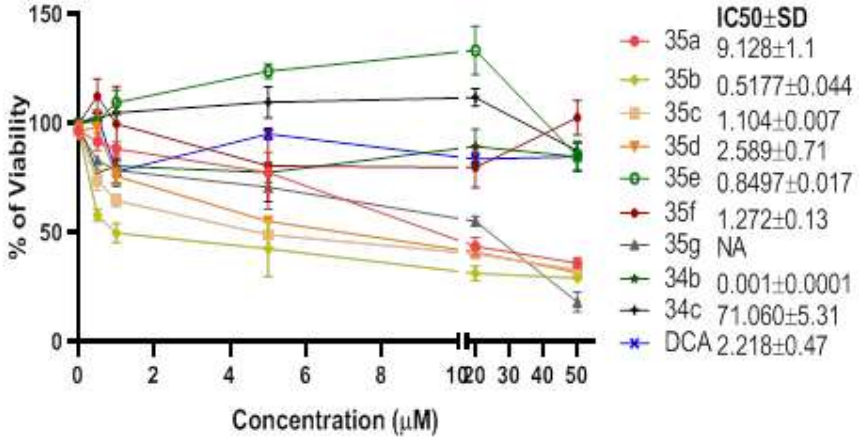
To the best of my knowledge, this is the first report on the synthesis of deoxycholic acid-chalcone conjugates, and the evaluation of their anticancer activity against hypertriploid human cell lines (SiHa), adenocarcinomic human alveolar basal epithelial cell lines (A549).

The chalcone group is modified at various positions in the ortho position and para positions which were further used to conjugate with bile acids. Based on side chain variability and associated charges, the resulting compounds may have different biological effects, which opens up the possibility of binding to various proteins involved in key biological functions in eukaryotic cells. The side chains with their hydrophilic nature also help in the uptake of the compound and retaining ability of the cells. Physiologically, bile acids aid in intestinal solubilisation and absorption of lipids, cholesterol and fat soluble vitamins. They are directly related to cytotoxicity based on their hydrophobicity and their ability to interact with the murine toxin X receptor (FXR) [16]. They have also been shown to have anticancer effects and induce death receptor-induced cytotoxicity in cancer cells[17]. However, cytotoxicity and apoptosis were reported at high concentrations. In the present study, we conjugated chalcones with bile acids to increase their efficacy against apoptosis and cell death.

Cytotoxic activity

Synthetic bile acid-chalcone amide conjugates (**35a-35g**) and their parent compounds were screened for cytotoxicity against A549 and SiHa cells. The inhibition of compounds was determined using the MTT assay. The anticancer activity was indicated in terms of IC_{50} (μM). The synthesised conjugate showed cytotoxicity with IC_{50} values ranging from (0.0001μM-71.06) for the SiHa cell line (**Figure 3.1A**) and (0.25-52.26μM) for the A549 cell line (**Figure 3.1B**). Cytotoxicity studies have shown that toxicity is cell line (type) dependent and highly variable.

Fig. 3.1A



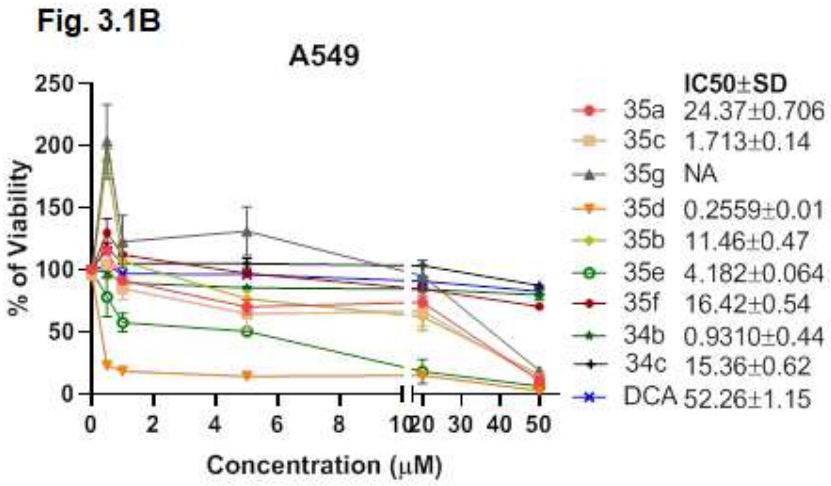


Figure 3.1: Cytotoxic effect of synthesized Bile-chalcone conjugates. Compounds (**35a-35g, 34b, 34c** and **DCA**) were dissolved in DMSO (10mM). Compounds were treated on confluent (a) SiHa and (b) A549 cell lines at different concentration for 48 h and analysed for cell viability by MTT assay. (n=3)

Deoxycholic acid was the least cytotoxic to the A549 cell line with an IC_{50} value of 52.26μM, while the chloro derivative of chalcone (**34c**) was the least cytotoxic to the SiHa cell line. Choloro-chalcone conjugate (**35c**) and hydroxy-chalcone conjugate (**3f**) compounds did not show any cytotoxicity against A549 and SiHa cell lines, respectively. Interestingly, the toxicity of choloro-chalcone (**34c**) alone was very low, while the toxicity of its bile conjugate was 1.1 μM.

For example, deoxycholic acid-chalcone conjugates **35b** (IC_{50}: 0.51 μM) and **35e** (IC_{50}: 0.84 μM) possessing o-nitro and p-3,4,5 trimethoxy groups showed fairly good activity against SiHa cancer cell line. Deoxycholic acid-chalcone conjugate **35d** (IC_{50}: 0.25 μM) with tolyl group showed excellent cytotoxicity against A549 cancer cell line. Compound **35b** showed good activity against both the cancer cell lines (IC_{50}: 0.84 μM for SiHa and IC_{50}: 1.73 μM for A549). It can be seen from the results that the nature of the substituents in the chalcone in the deoxycholic acid-chalcone conjugate plays a key role in inhibiting the growth of cancer cells. Most of the chalcone derivatives tested were not inherently toxic, but the bile conjugates were toxic to the cancer cell lines tested. This suggests that the conjugation of bile acids to chalcone will be beneficial and improve the cytotoxicity of bile acids.

Fig. 3.3 A

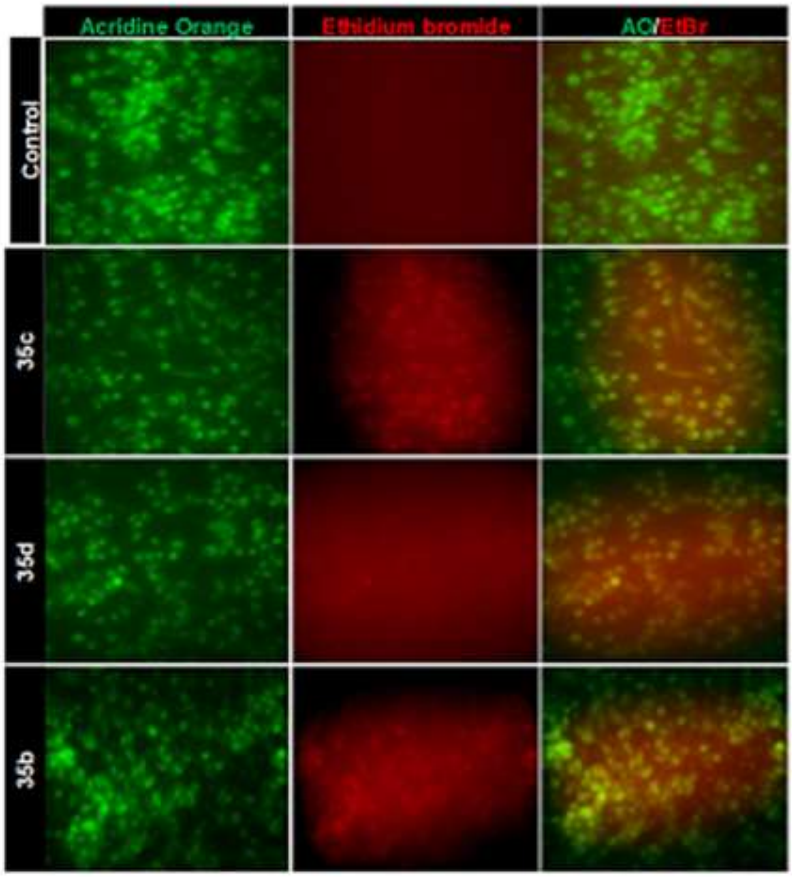


Fig. 3.3 B

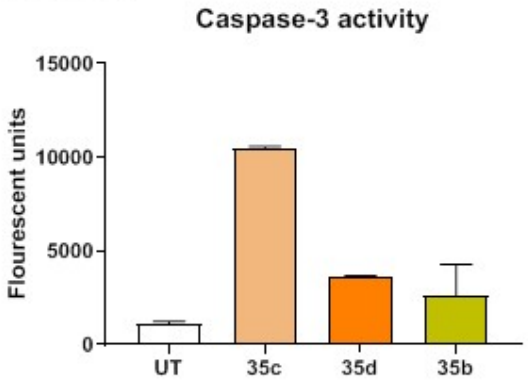


Figure 3.3: Chalcone-bile conjugates induce apoptosis in cancer cells. (A) SiHa cells were treated with **35c, 35b** and **35d** for 24 h, stained with acridine orange-propidium iodide and observed under fluorescence microscope. (B) In a replicate experiment after incubation for 24 h with indicated compounds, cells were lysed and analyzed for caspase-3 activity and fluorescence units were plotted representing rate of caspase-3 substrate cleavage. (n=3; *** represents $p<0.01$).

Bile-conjugates which were showing promising cytotoxicity against the tested cancer lines were further analysed (for cell death mechanism apoptotic/necrosis) using acridine orange/PI staining (**Figure 3.3A**). The increase in the intensity and distribution of Et-Br denotes apoptotic to necrotic cell death. Acridine orange (AO)/ethidium bromide (EtBr) staining can be used to identify apoptosis-associated changes. AO can penetrate normal and early apoptotic cells, and its intact membrane fluoresces green when bound to DNA. EBs cannot enter intact cells. EBs can only bind to DNA in damaged membranes during late stages, stages of apoptosis, and when dead cells fluoresce orange-red. Thus, normal and early apoptotic cells show green fluorescence, and late apoptotic and dead cells emit green fluorescence[53]. Synthesized compounds show varying degrees of orange fluorescence indicating apoptosis, as evidenced by caspase-3 activity

Bile acids conjugated with nitro-, chloro- and tolyl chalcone exhibited apoptotic ability on SiHa cell line. Chloro- and nitro chalcone conjugates showed early apoptosis after 48 h (Et-Br-stained cells). The apoptotic ability of these compounds is beneficial for various pathological conditions and can be further exploited for anticancer effects in mouse models. The apoptosis-inducing abilities of best performing compounds (**35b, 35d** and **35e**) was further confirmed with an increase in the activity of caspase-3 in the cells, which is an apoptosis executioner caspase in the eukaryotic cells (**Figure 3.3B**).

The presented results indicate the bile acids conjugates induced apoptosis in cancer cell lines and can be further explored for its anti-cancer effects. Further studies are warranted to explore the molecular mechanism and efficacy in mice models of cancer.

The following observations could be inferred from our studies:

- Lower IC_{50} values of deoxycholic conjugate (**35b**, **35d** and **35e**) directed a crucial role of substituents in the chalcone skeleton in inhibiting the growth of SiHa and A549 cancer cell lines.
- Deoxycholic acid and chalcone derivatives do not exhibit any anticancer activity whereas some of their deoxycholic acid-chalcone conjugates displayed good anticancer activity. This outcome suggesting that the importance of deoxycholic acid molecules on the activity of conjugates.
- Amongst the deoxycholic acid-chalcone derivatives, the presence of the 3, 4, 5-methoxy group in the chalcone moiety yielded a comparatively better activity. For instance, **35d** (IC_{50}: 0.25 μM; 3, 4, 5-methoxy group) was found to be most active against A549 cancer

cell line as compared other conjugates. Similarly, **35b** (IC_{50}: 0.51 μM; nitro group) was found to be more active against SiHa cancer cell line as compared other conjugates.

However, these observations cannot be generalized for all molecules. The potential conjugates (**35b**, **35d** and **35e**) will be further explored for their task in the growth inhibition of other human tumour cells. Thus, steroidal chalcones may be developed as potent anticancer agents. The current research has provided some insights on its further optimization for better activities

Antioxidant activity

The results of the antioxidant activity can be seen in **Table 3.1**. Among all conjugates, conjugate **35f** showed antioxidant at 15.04 μM in DPPH assay and at 85.92 in ABTS assay. Additionally, this requirement was lower than that indicated by the reference compound (ascorbic acid). Compound **35e** (IC_{50} 15.04 mM) also exhibited better antioxidant activity in DPPH assay than reference compound. Compound **35f** has a phenolic group, which may be responsible for the absorption of the radical DPPH, exhibited better antioxidant activity than reference molecules (ascorbic acid). While the conjugate, **35g**, showed the best antioxidant activity (67.97 μM) amongst all the compounds in the ABTS assay. The choice for adding chalcones was based on the fact that polyphenolic compounds chalcone is an important intermediate in the synthetic pathway of flavonoids and has been shown to possess various biological and pharmacological activities, such as anticancer, antioxidant, anti-inflammatory, etc. It also plays a role in neutralising free radicals and chelating transition metals, both in the initiation step and in the propagation of oxidative stress.

Table 3.1: *Antioxidant activity of synthesized conjugates with DPPH and ABTS assay*

No	Entry no	IC50 (μg/ml)		Entry no	IC50 (μg/ml)	
		DPPH	ABTS		DPPH	ABTS
1	35a	29.52	73.84	34a	148.33	387.61
2	35b	70.74	92.30	34b	-	-
3	35c	24.65	204.09	34c	139.06	215.73
4	35d	63.53	98.06	34d	259.43	208.41
5	35e	15.04	85.92	34e	144.74	251.68
6	35f	11.73	97.72	34f	84.54	179.11
7	35g	50.10	67.97	34g	125.85	180.27
8	Ascorbic Acid	20.72	56.01	DCA	128.95	107.96

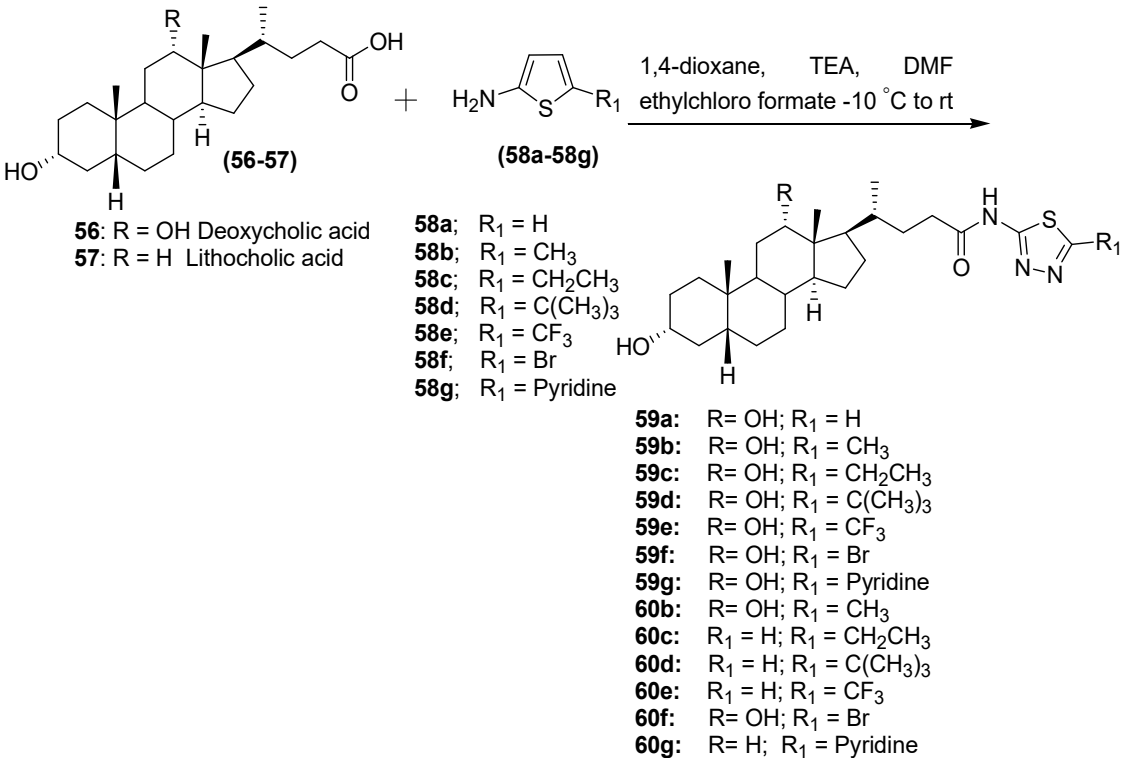
In conclusion, we have synthesized a series of novel 4-amino chalcone derivatives, and their deoxycholic acid conjugates in excellent yields as an anticancer agent. Synthesized conjugates were evaluated for their cytotoxicity against two cancer cell lines. The outcomes of this study displayed that the bile acid–chalcone conjugates can be an inhibitor of cancer cell lines human cancer cell-lines A549 and SiHa. Based on the current results, further studies of these conjugates on other cancer cell lines are warranted. Thus, sterol-chalcones may be developed as potent anticancer agents. Compound **35f** showed better antioxidant activity than reference molecules in DPPH assay. The current study provides some insights into its further optimization for better activity.

Chapter 4: Synthesis of Bile Acid-Thiadiazole Conjugates as Antibacterial and Antioxidant Agents

Chapter 4 describes the synthesis of bile acid-thiadiazole conjugates and their biological evaluation. Thiadiazole rings are a versatile scaffold that has been extensively studied in medicinal chemistry. Subsequently, these compounds have a wide range of biological activities. Due to poor bioavailability, they are rapidly eliminated from the bloodstream without function, and only a fraction of the administered drug can be delivered to the target site. The bioavailability and chemo sensitivity of thiadiazole are increased by attachment to hydrophilic molecules. Hence, this approach was introduced into the development of new thiadiazole-based therapeutics. In this work, we focused on the conjugation of these steroids with thiadiazole. The rationale for this bile acid-thiadiazole conjugate approach was based on the expectation that in these conjugates bile acid parts would be useful to permeabilize the cell membrane as well as the transporter of the pharmacophore of thiadiazole because of its amphiphilic nature and hence the conjugates were predicted to be more active than thiadiazole itself. Consequently, bile acids improve the bioavailability of drugs that have either poor aqueous solubility or low membrane permeability. Considering this, we plan to synthesise bile acid conjugates of 1, 3, 4-thiadiazoles to obtain better inhibitors, increase drug activity and reduce toxicity.

Synthesis of the conjugate (**Scheme 4.1**) starts with commercially available bile acids and thiadiazoles. For the synthesis of **59a-59g** and **60a-60g**, the first step was to convert the corresponding deoxycholic and lithocholic acids into activated anhydride form via the reaction of deoxycholic and lithocholic acids with ethyl chloroformate and triethyl amine in THF and DMF solutions at 0°C for 20 minutes. After 20 min, 2-amino thiadiazole/substituted 2-amino thiadiazole (**58a-58g**) and triethylamine were added to the reaction mixture as a

solution in DMF. The reaction mixture was stirred until the completion of the reaction. After the completion, the crude product was purified using silica gel chromatography with ethyl acetate and hexane as eluent to furnish the deoxycholic acid-thiadiazole conjugates **59a–59g** and **60a-60g** in 60–65% yields. The structures of the synthesized bile acid-chalcone conjugates were characterized employing ^{1}H & ^{13}C NMR and FTIR spectroscopic studies..


1,4-dioxane, TEA, DMF
ethylchloro formate -10 °C to rt
(56-57)
(58a-58g)
56: R = OH Deoxycholic acid
57: R = H Lithocholic acid
58a; R1 = H
58b; R1 = CH3
58c; R1 = CH2CH3
58d; R1 = C(CH3)3
58e; R1 = CF3
58f; R1 = Br
58g; R1 = Pyridine
59a: R= OH; R1 = H
59b: R= OH; R1 = CH3
59c: R= OH; R1 = CH2CH3
59d: R= OH; R1 = C(CH3)3
59e: R= OH; R1 = CF3
59f: R= OH; R1 = Br
59g: R= OH; R1 = Pyridine
60b: R= OH; R1 = CH3
60c: R1 = H; R1 = CH2CH3
60d: R1 = H; R1 = C(CH3)3
60e: R1 = H; R1 = CF3
60f: R= OH; R1 = Br
60g: R= H; R1 = Pyridine

Scheme 4.1: Synthesis deoxycholic/lithocholic acid–thiadiazole conjugates (**59a-59g; 60a-60g**)

Antioxidant Activity:

The antioxidant activities of the parent 1, 3, 4-thiadiazole and its conjugates with DCA and LCA were investigated by two free-radical assays, DPPH and ABTS, and the results are shown in **Table 4.1**. Ascorbic acid was used as an antioxidant standard. Antioxidative assessments were performed using synthetic conjugates and starting substrates at different concentrations (100, 50, 25, 12.5 and 6.25 μM). The antioxidant activity of this compound depends on the ability to release protons and the ability of the functional group attached to the 1, 3, 4-thiadiazole to form stable free radicals.

The IC_{50} of antioxidant activity data indicated that thiadiazole-deoxycholic/lithocholic acid conjugates and its derivative **59b, 59c, 59d, 59e, 60a, 60b, 60c,** and **60g** exhibited the good antioxidant activity with IC_{50} values of 13.45–19.23 μg/mL compared to ascorbic acid (20.9 μg/mL) in DPPH assay.

Table 4.1 shows a comparison between the IC50s of the tested compounds. Most of the thiadiazole and bile acid conjugates showed better activity than standard ascorbic acid. It is evident from the antioxidant activity that conjugates **59c** and **60c** showed good DPPH and ABTS detection activities with IC_{50} at 15.34 and 13.45 (μg/ml) for DPPH assay and 67.65 and 69.18 (μg/ml) for ABTS assay, respectively. The **59c** conjugate showed superior activity against ABTS at a concentration of 69.65 (μg/ml). In the present study, the highest DPPH radical-scavenging effects were observed for the **60c**, with an inhibition value of (IC_{50} 13.45 μM). The introduction of an ethyl group at the 5-position to thiadiazole moiety increased the activity to a significant extent in the DPPH model. The hybrid molecules of thiadiazole and bile acid (deoxycholic acid/lithocholic acid) exhibited better antioxidant activity than bile acid and thiadiazole. The consequences of the results validate the importance of bile acids in the conjugate.

Table 4.1: Antioxidant activity of derivative of 1, 3, 4-thiadiazole, deoxycholic acid/lithocholic acid and the conjugates of 1, 3, 4-thiadiazole- deoxycholic/lithocholic acid

Entry	IC50 (μg/ml)		Entry	IC50 (μg/ml)	
	DPPH	ABTS		DPPH	ABTS
59a	21.81	83.27	60h	28.30	83.54
59b	16.49	74.87	58a	75.84	171.56
59c	15.34	69.65	58b	61.09	152.47
59d	17.70	85.51	58c	56.02	109.75
59e	19.23	87.11	58d	74.08	105.53
59g	31.19	78.53	58e	62.78	142.56
59f	22.03	83.27	58f	63.06	107.92
59h	26.12	87.66	58g	67.22	108.13
60a	19.93	93.19	58h	72.56	152.64
60b	17.19	83.54	56	128.95	107.96
60c	13.45	67.59	57	135.34	127.30
60f	22.32	77.63	Ascorbic Acid	20.72	56.01
60g	18.01	83.54			

Studies have shown that the 5-position substitution with an electron donor such as methyl, ethyl, tert-butyl, etc., increases the antioxidant activity of thiadiazole-deoxycholic acid conjugates by enhancing the stability of thiadiazole-deoxycholic acid conjugates radical

by inductive effect and enhances the facile transfer of the hydrogen atom. However, when the 5-position is substituted with an electron-withdrawing group such as a CF_3 group, as shown in compound **59e**, the activity of both the DPPH and ABTS models is significantly reduced.

Antibacterial activity*:*

The synthetic molecules screened showed differential inhibition against the Gram-positive and Gram-negative bacterial strains tested (**Table 4.2**). Conjugates (**59c** (IC_{50} 0.015 mM)**, 59g** (IC_{50} 0.082 mM) and **59c** (IC_{50} 0.020 mM)) were found to have good activity against B. megaterium, whereas conjugates **4e** (IC_{50} 0.872 mM) and **5f** (IC_{50} 0.652 mM) were found to be less active against *B. megaterium*. Compounds **59b, 59d, 59g** and **60b** exhibited similar activities against *B. megaterium* and *B. subtilis*. However, the IC50 of the *B. megaterium* strain was lower than that of *B. subtilis*. Conjugates **59c** (IC_{50} 0.024 mM)**, 59g** (IC_{50} 0.029 mM)**,** and **60c** (IC_{50} 0.093 mM) were most effective against Gram-negative bacteria (*P. vulgaris*), while conjugates **59e** (IC_{50} 0.055 mM) and **5f** (IC_{50} 0.052 mM) were most effective against Gram-negative bacteria (*E. coli*).

Table 4.2: Antibacterial activity (IC50) of deoxycholic acid/lithocholic acid-thiadiazole conjugates against Gram positive (*B. megaterium, B. subtilis*) and Gram positive (*E. coli, P. vulgaris*)

Compound No.	Gram positive *(IC_{50}* mM*)*		Gram negative*(IC_{50}* mM*)*	
	B. megaterium	*B. subtilis*	*E. coli*	*P. vulgaris*
59a	0.633	0.752	0.934	1.26
59b	0.142	0.206	0.641	0.544
59c	0.015	0.081	0.181	0.024
59d	0.225	0.257	0.415	0.486
59e	0.872	0.827	0.055	0.964
59g	0.082	0.045	0.130	0.029
59h	0.537	0.485	0.651	0.892
60a	0.531	0.297	0.562	0.778
60b	0.354	0.553	0.813	0.914
60c	0.020	0.051	0.132	0.093
60f	0.654	0.755	0.052	0.801
60h	1.130	0.857	0.730	0.845
56	1.180	1.310	0.810	0.780
57	1.240	1.580	0.905	0.812

The starting precursor's deoxycholic acid/lithocholic acid and thiadiazole derivatives exhibited antibacterial activity at concentrations above 1 mM. In general, most of the compounds examined have strong antibacterial effects against Gram-positive rather than Gram-negative bacteria. Furthermore, compounds **59c, 59e** and **59c** showed the greatest antibacterial activity compared to all tested compounds. None of the synthetic compounds showed better inhibitory activity than standard drugs.

Conjugates with two carbon alkyl groups (**59c, 60c**) showed good activity against *B. megaterium, B. subtilis* and *P. vulgaris*. Whereas one carbon alkyl group (**59b, 60b** methyl) and four alkyl tert butyl groups (**59d**) substituted deoxycholic/lithocholic acid thiadiazole conjugates showed lesser activity than two carbon alkyl groups (**59c, 60c**). The results show that electron donating groups with two carbon alkyl groups have the best antibacterial activity. It was found that the most potent was compound **59c** and **60c**, which had the electron donating group (ethyl) exhibits good activity against gram positive bacteria (*B. megaterium, B. subtilis*). The presence of electron withdrawing shows good activity against the Gram-negative bacterium *E. coli* but poor activity against *B. megaterium, B. subtilis and P. vulgaris*. However, conjugates **59a**, **59b, 59c, 59d, 60a, 60b** and **60c** showed weaker antibacterial activity compared to the active compounds **59e** and **60f** against *E. coli.*

By comparing the obtained results (**Table 4.1** and **4.2**) with the conjugated structures, the following SARs can be derived: (i) Compounds containing a donor group (**59c**, **60c** with two carbons) more efficient than compounds with and without a donor group against gram positive bacteria (*B. megaterium, B. subtilis*). (ii) The presence of electronegative atoms (**59e**, **60f**) increases the antibacterial activity against *E. coli*. (iii) The presence of the aromatic tertiary amine pyridine (**59g**) inhibited *Bacillus megaterium, Bacillus subtilis* and *B. vulgaris* at lower concentrations. (iv) All deoxycholic acid/lithocholic acid-thiadiazole conjugates were more active than deoxycholic acid/lithocholic acid and thiadiazole derivatives. (v) The introduction of an ethyl group at the 5-position to thiadiazole moiety increased the activity to a significant extent in the DPPH model. The hybrid molecules of thiadiazole and bile acid (deoxycholic acid/lithocholic acid) exhibited better antioxidant activity than bile acid and thiadiazole. The consequences of the results validate the importance of bile acids in the conjugate.

In conclusion, novel series of thiadiazole-bile acid conjugates containing amide were designed and synthesized. These molecules were tested for their antioxidant and antibacterial activity. Out of these compounds containing ethyl group showed very good antibacterial activity against *B. megaterium, B. subtilis* and *P. vulgaris*. Some of the synthesized compounds

showed better antioxidant activity than the reference compound ascorbic acid in DPPH and ABTS assays. The *in vivo* assay also indicated that these lead molecules may good candidates for drug design. Thus a large library of compounds can be easily synthesized from the bile acid for extensive structure–activity relation studies so that one can reach to the more appropriate drug candidate.

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